Biostimulation of Iron Reduction and Uranium Immobilization: Microbial and Mineralogical Controls

Joel E. Kostka[1], Heath Mills[1], Denise Akob[1], Thomas Gihring[1], Joseph W. Stucki[2], Bernard A. Goodman[2], and Lee Kerkhof[3]

[1] Department of Oceanography, Florida State University, Tallahassee, FL, 32306
[2] Natural Resources and Environ. Sciences Department, Univ. of Illinois, Urbana, IL
[3] Inst. of Marine and Coastal Sciences, Rutgers Univ., New Brunswick, NJ

Abstract

Our overall objective is to understand the microbial and geochemical mechanisms controlling the reduction and immobilization of U(VI) during biostimulation in shallow subsurface sediments cocontaminated with uranium and nitrate. The focus is on the activity and community composition of microbial populations (metal- and nitrate-reducing bacteria) and iron minerals that are likely to make strong contributions to the fate of U during *in situ* bioremediation. Our integrated approach was applied to sediment cores and microcosms of site materials from Area 2 of the Field Research Center (FRC) at Oak Ridge, TN. Substantial differences were observed in the abundance and activity of microbial groups depending upon the electron donor (glucose or ethanol) used for biostimulation. Viable counts revealed that Fe(III)- and nitrate-reducers are abundant (10^4 to 10^5 per g wet) in Area 2 sediments and counts were shown to be carbon substrate dependent. U(VI) and Fe(III) were reduced concurrently in the glucose but not the ethanol treatments. One to 2 orders of magnitude more Fe(III)-reducers were observed in ethanol- as compared to glucose-amended treatments in parallel with enhanced U(VI) removal in ethanol treatments. Cultivatable Fe(III)-reducing bacteria in the ethanol treatments were numerically dominated by *Geobacter* sp. while those cultured on glucose were dominated by fermentative organisms. Efforts are underway to associate *in situ* activity at the FRC with the bacteria from the microcosms by fingerprinting ribosomes in groundwater samples.

Iron minerals were characterized by Mössbauer spectroscopy over a wide range in temperature (4 to 298 K) in order to fully determine the form and speciation of Fe. Spectra at room temperature (298 K) exhibited no sextet pattern, thus excluding the presence of hematite, magnetite, and maghemite. At 77 K, the amount of Fe(II) doubled from 15 to 30 % in ethanol- and glucose-amended relative to unamended microcosm sediments, in parallel with wet chemical extractions and counts of Fe(III)-reducing bacteria. Poorly ordered or Al-substituted goethite was identified and appeared to be dissolved by microbial activity. However, silicate bound Fe(III) clearly predominated over the Fe minerals reduced.

Novel iron(III)- and sulfate-reducing organisms were isolated from the contaminated FRC subsurface that shared high sequence identity (96 to 99%) to *Geobacter bremensis* and *Desulfotomaculum ruminis*, respectively. The *Desulfotomaculum*-related isolate utilizes Fe(III) as well as sulfate as an electron acceptor. The draft genome sequence of *Geobacter* strain FRC-32 has been completed by the Joint Genome Institute and annotation is currently underway. Our results have the following implications for U bioremediation in the FRC subsurface: 1) the microbially-catalyzed mechanism of U(VI) reduction is electron donor dependent, 2) silicate bound Fe is an important oxidant that is transformed by indigenous microbial populations in the Area 2 subsurface, and 3) *Geobacter* sp. predominate over other Fe(III)-reducing bacteria during biostimulation with ethanol as an electron donor.

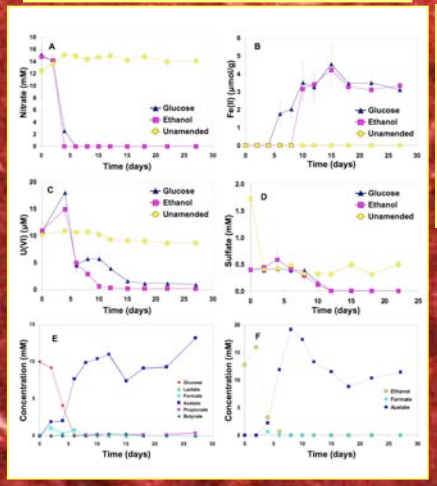
Approach

Microcosms
-Area 2 sediment (FB094) was combined with Area 2 groundwater (FW209).
-Microcosms were sealed, neutralized and flushed with N_2.
-Treatments (3 replicates each): 20 mM Ethanol, 10mM Glucose, Unamended control
-Incubated at 30°C and sampled for geochemical analysis (nitrate, Fe(II), & U(VI)) every 1-5days.

Microbial Community Analysis
Cultivation-Dependent Community Analysis
-A most probable number (MPN) dilution series was used to enumerate nitrate- and iron-reducing bacteria present in the microcosms and FB094 sediment.
-After 3 months of growth the highest positive dilutions were sampled for NO_3- and Fe(III)-reduction, HPLC and molecular analysis (SSU rRNA).

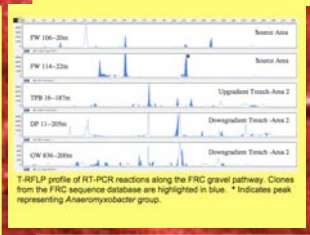
Cultivation-Independent Community Analysis
-DNA was extracted from microcosm samples and MPN cultures.
-Cloning and sequencing of PCR amplified SSU rRNA genes.
-Fingerprinting using terminal restriction fragment length polymorphism (TRFLP).
-SSU rRNA genes were PCR amplified with a fluorescently labeled 27F primer
-PCR products were digested with restriction enzyme *Mnl*I

Microcosms

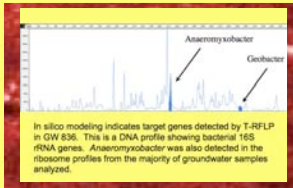


Electron flow in microcosms of Area 2 sediments. Values are the averages of triplicate incubations: (a) nitrate, (b) Fe(II), (c) U(VI), (d) sulfate, (e) HPLC for glucose treatment, and (f) HPLC for ethanol treatment

Microbial Community Analysis



T-RFLP profile of RT-PCR reactions along the FRC gravel pathway. Clones from the FRC sequence database are highlighted in blue. * Indicates peak representing *Anaeromyxobacter* group.

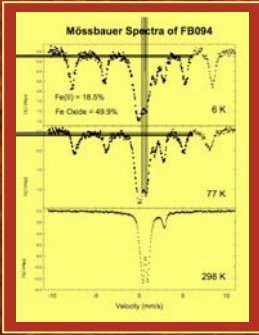


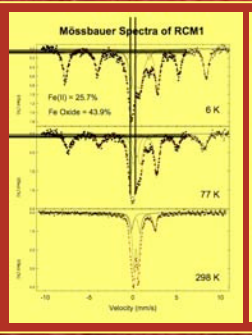
In silico modeling indicates target genes detected by T-RFLP in GW 836. This is a DNA profile showing bacterial 16S rRNA genes. *Anaeromyxobacter* was also detected in the ribosome profiles from the majority of groundwater samples analyzed.

Isolates

- ***Geobacter* strain FRC-32**
  - Phylogenetic analyses of 16S rRNA genes identify FRC-32 as a novel lineage within the *Geobacteraceae*.
  - FRC-32 reduced solid-phase iron without an electron shuttle.
  - The optimal pH was ~ 6 with no growth detected at pH 5.
  - A limited suite of electron donors were utilized.
  - Growth was not detected with nitrate and sulfate as electron acceptors.
- ***Desulfotomaculum* sp.**
  - Isolates 27C1 and 30205 were obtained from contaminated and background sediment, respectively, and share >99% 16S rRNA sequence identity.
  - *Desulfotomaculum* spp. 27C1 and 30205 grew optimally with lactate and sulfate.
  - Reduction of FeOOH with AQDS as an electron shuttle was also demonstrated.
  - Ethanol, hydrogen, lactate, and malate were utilized as electron donors and not acetate, citrate, fumarate, propionate, or succinate.
- ***Anaeromyxobacter* sp.**
  - In collaboration with Sara Thomas and Frank Loeffler (Georgia Tech), several isolates were obtained from highly contaminated sediments of Area 1

Iron Mineralogy





Mössbauer Parameters at 6 K

Sample	Phyllosilicate Peak Areas		Goethite Field & Peak Areas		Chemical Analysis*
	$A_{Fe(II)}$ %	$A_{Fe(II)}$ %	B_{hf} T	A %	Fe(II) % of tot Fe
FB094	31.6	18.5	49.67	49.9	13.1
RCM7	37.8	12.9	49.72	49.3	12.5
RCM4	24.0	20.3	49.63	55.7	24.0
RCM1	30.3	25.7	49.83	43.9	31.2

*1,10-phenanthroline method of Komadel and Stucki, Clays & Clay Minerals 36:379-381.

SAMPLE DESIGNATIONS: FB094 – untreated, homogenized sediment; RCM 7, unamended microcosm; RCM 4, ethanol amended; RCM 1, glucose amended.
MÖSSBAUER SPECTRA: obtained using a Web Research, Inc. spectrometer equipped with a Janis Model SHI-850-5 Closed Cycle Cryostat, operating at a sample temperature of 6 to 298 K.

Conclusions

Microcosms/ Community Analysis

- The mechanism of U(VI) reduction is electron donor dependent, with substantial reduction occurring prior to Fe(III) reduction in microcosms
- Carbon utilization defined by fermentative metabolism and incomplete oxidation of ethanol
- *Geobacter* sp. numerically dominate cultivatable Fe(III)-reducing bacteria in ethanol microcosms
- However, only *Anaeromyxobacter* is detected in the metabolically active profiles of highly contaminated groundwaters collected near FRC source zone
- Highly impacted, upgradient groundwater samples had roughly half the T-RFLP peaks of downgradient samples suggesting lower diversity in contaminated groundwaters
- A database of > 1400 16S rRNA gene sequences retrieved from FRC materials is now available and > 400 have been examined with *in silico* digestion for T-RFLP profiling
- Please see Denise Akob's poster for microbial community analysis of microcosm samples!

Isolates

- Pure cultures have been obtained from FRC subsurface sediments for at least four groups of Fe(III)-reducing bacteria (*Geobacter, Desulfotomaculum, Anaeromyxobacter, Clostridium*)
- *Geobacter* and *Desulfotomaculum* strains are physiologically distinct from close relatives. The *Desulfotomaculum* isolate is capable of growth using both Fe(III) and sulfate
- The genome sequence of *Geobacter* strain FRC-32 is now available from JGI
- In collaboration with the Loeffler lab (Georgia Tech), several *Anaeromyxobacter* strains were isolated; these have yet to be characterized

Iron Mineralogy

- Mössbauer analysis shows that phyllosilicates and goethite predominate
- The following are estimated for goethite: a surface area of 42.5 m^2/g, isomorphous Al substitution of 16.2%, and a mean crystallite diameter (MCD) of 32.8 nm
- Mössbauer supports wet chemical analysis to show

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